each side fuses with its opposing medial nasal prominence, while the medial nasal prominences grow and merge together in the midline (Figs. 8E and F). In this manner, the upper lip and alveolar (tooth) ridge are formed with the incisor portion and primary palate derived from the medial nasal prominences and the remainder of the upper lip and jaw formed by the maxillary prominences (Fig. 8F). The lateral nasal prominences form the alae (sides) of the nose (Adapted from Langman's Medical Embryology, 12th ed.).

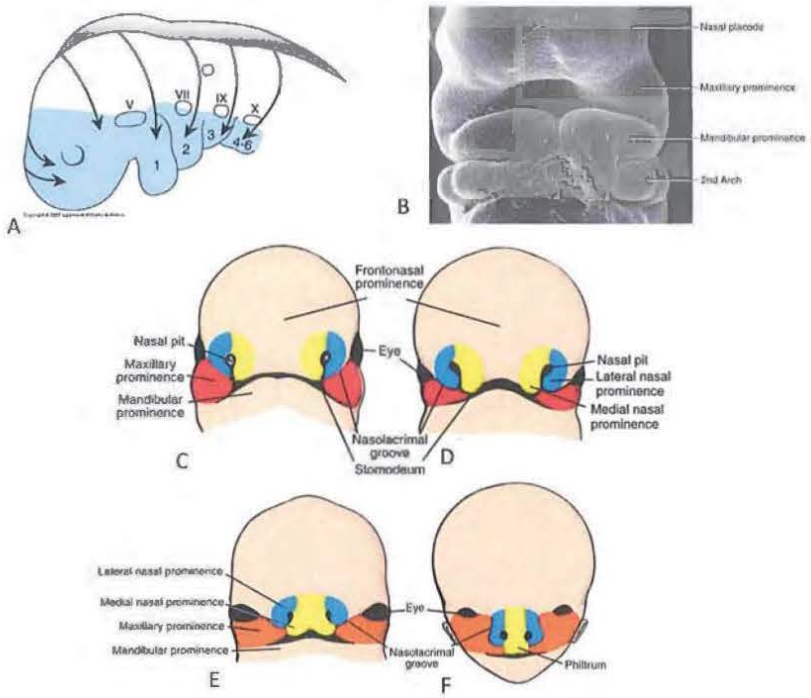


Figure 8. Neural crest cells (NCC), originating in cranial neural folds, migrate to the facial region and create the facial prominences, including, the frontonasal, maxillary, and mandibular prominences (**A** and **B**). Nasal placodes form bilaterally and then invaginate to form the nasal pits creating the medial and lateral nasal prominences surrounding the pits (**B-D**). With growth of the maxillary and medial nasal prominences, a line of fusion is formed between these 2 structures (**D** and **E**). Continued growth and merging of the medial nasal prominences forms the philtrum of the upper lip, the primary palate, and the part of the alveolar ridge containing the 4 incisor teeth (**F**). Lateral portions of the upper lip and alveolar ridge are formed by the maxillary prominences, that fuse completely with the medial nasal prominences (**F**). **A**. 3 weeks; **B**. 4.5 weeks; **C**. 5 weeks; **D**. 6 weeks; **E**. 7 weeks; **F**. 10 weeks.

In addition to forming much of the lip and alveolar ridge, maxillary prominences are also responsible for forming the secondary palate. By week 6 of gestation, palatine shelves form on each side of the tongue from expansions of the maxillary prominences into the oral cavity. These shelves are suspended vertically and are widely separated by the tongue (Figs. 9A and B). With

growth of the mandible, the tongue moves ventrally, allowing the palatal shelves to re-orient into a horizontal position above the tongue (Figs. 9C and D). The shelves then grow toward the midline and fuse to form the secondary palate (Fig. 10A).

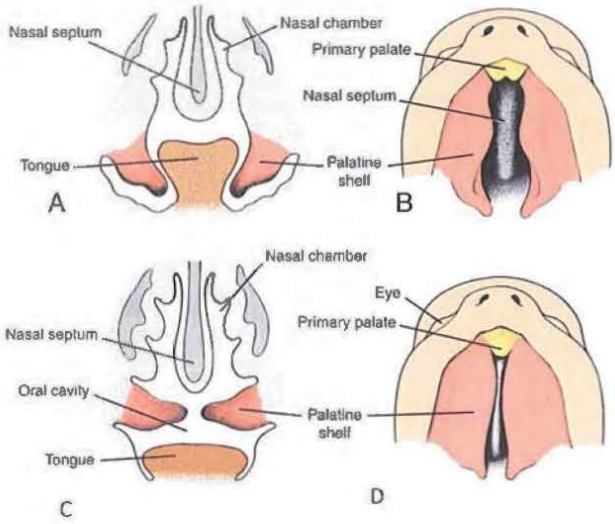


Figure 9. Formation of the secondary palate. **A**. Cross section through the oral and nasal cavities showing the vertical positioning of the palatal shelves on either side of the tongue, which keeps the shelves widely separated at 6.5 weeks (**B**). **C**. Cross section through the oral and nasal cavities showing opposing re-oriented palatal shelves at 7.5 weeks, which are separated by a narrower space after elevation (**D**). From this horizontal position the shelves grow together and fuse in the midline by 10 weeks. The primary palate (yellow) is formed by the medial nasal prominences.

Clefts of the lip and/or palate are illustrated in the drawings and pictures in Figure 10. Cleft lip may involve only the lip (Figs. 10 B and C) or may include the alveolar ridge (jaw) as well (Figs. 10), and may be unilateral (Fig. 10D) or bilateral (Figs. 10E and F). Cleft palate may appear as an isolated defect (Fig. 10G and I) or in combination with cleft lip (Fig. 10H). These clefting defects arise in multiple ways: NCC fail to form the aforementioned prominences; growth of the prominences fails; fusion between prominences fails; or palatal shelves fail to re-orient. (Adapted from Langman's Medical Embryology, 12th ed.) SSRIs including Zoloft can perturb each of these developmental processes resulting in congenital cleft lip and palate defects.

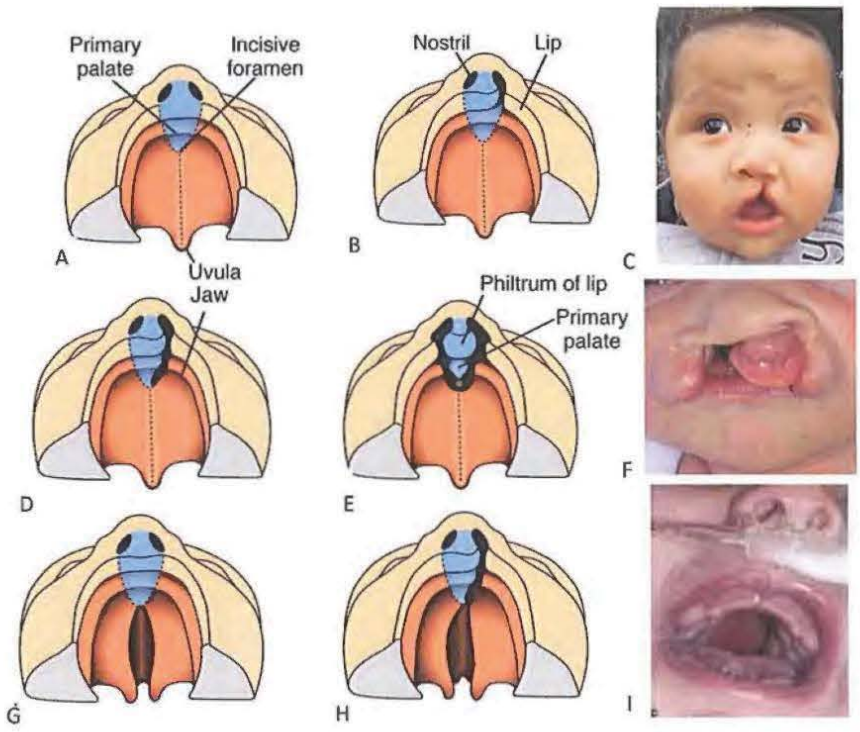


Figure 10. A. Normal lip and palate development; **B** and **C**. Cleft lip without involvement of the alveolar ridge; **D**. Unilateral cleft lip and alveolar ridge; **E** and **F**. Bilateral cleft lip and alveolar ridge; **G**. Isolated cleft of the secondary palate; **H**. Cleft of the lip and secondary palate; **I**. Isolated cleft of the secondary palate.

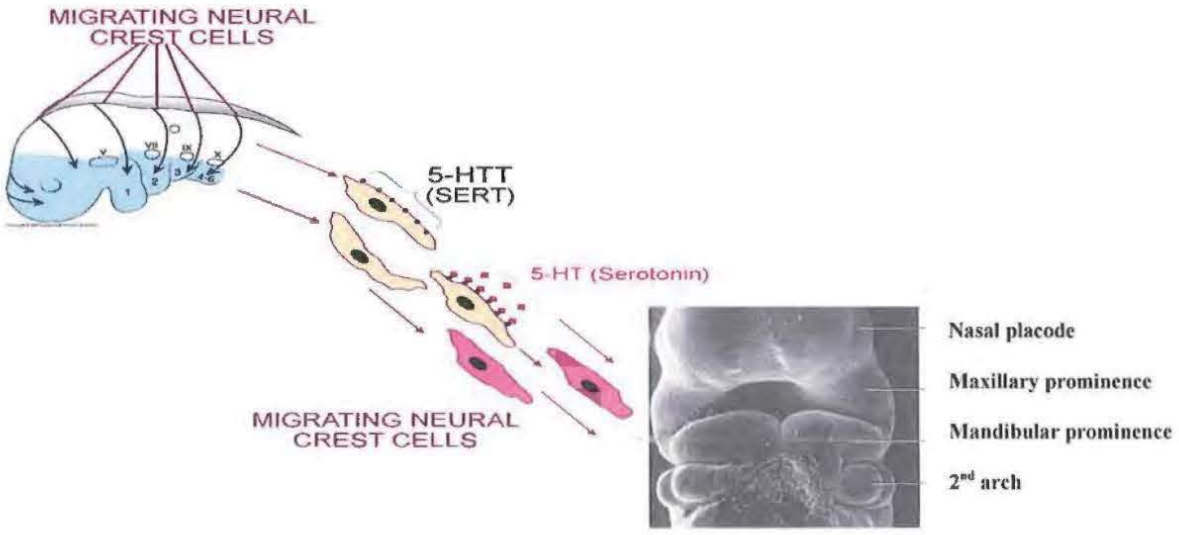


Figure 11. Neural crest cells (NCC) migrate from the edges of the cranial neural folds into the facial region (blue) to form the prominences that grow to become the different parts of the face. NCC express 5-HT receptors and the 5-HT transporter SERT.

B. SSRIs Including Zoloft Adversely Affect Neural Crest Cells Involved in Lip and Palate Development

Neural crest cells (NCC) are the key to facial development because they form the facial prominences, including the nasal and maxillary prominences that form the upper lip and jaw and the secondary palate (Fig. 11; Johnston, '66; LeDouarin, '82; Noden, '84, '88; Tam and Morriss-Kay, '85; Nichols, '86). Just as these cells are essential to septation of the outflow tract in the heart (See Section **VI**), NCC are essential for normal development of the face. In this regard, it is well established that interference with the migration, proliferation, viability, or differentiation of these cells can result in craniofacial defects, including cleft lip and/or palate (Webster et al., '86; Bolande, '97). NCC express 5-HT receptors (Moiseiwitsch and Lauder, '95; Choi et al., '97) and the 5-HT transporter SERT (Hansson et al., '99), such that 5-HT is an important signal molecule that regulates NCC migration and viability (Fig. 11; Moiseiwitsch and Lauder, '95; Choi et al., '97). Thus, one teratogenic mechanism whereby SSRIs like Zoloft can cause cleft lip and palate is by disrupting normal NCC participation in the initial stages of lip and palate embryogenesis when the maxillary and nasal prominences are forming.

C. SSRIs Including Zoloft Interfere with Epithelial-Mesenchymal Interactions

Epithelial-mesenchymal interactions are a fundamental requisite for normal embryogenesis of many structures (Grobstein, '67; Holtfreter, '68; Noden, '83). In the craniofacial region, interactions between neural crest derived mesenchyme cells and surface epithelia maintain growth and differentiation of the facial processes, including those of the lip and palate (Bee and Thorogood, '80; Hall, '81; Noden, '83). 5-HT appears to be involved in these types of interactions. For example, epithelia covering the nasal processes and palatal shelves have multiple uptake sites for 5-HT (SERT) and this uptake is transient and correlated with rapid growth of these structures (Lauder and Zimmerman, '88; Lauder et al., '88; Shuey et al., 92). SSRIs including Zoloft block 5-HT uptake in these cells causing decreased cell proliferation and increased cell death (apoptosis) in underlying mesenchyme cells (Shuey et al., '92), indicating that SSRIs like Zoloft disrupt normal epithelial-mesenchymal interactions in these structures. Also, extracellular 5-HT binding protein (SBP) is present in the mesenchyme (Lauder et al., '88). Initially, SBP is distributed throughout the mesenchyme of the facial prominences, but then, as epithelial-mesenchymal signaling is initiated, the protein is

redistributed and becomes localized around mesenchyme cells adjacent to 5-HT uptake SERT sites in the overlying epithelium (Shuey, 91). SBP functions as a storage protein for 5-HT (Tamir and Gershon, '79) and may be important for modulating 5-HT concentrations during signaling between the epithelium and underlying mesenchyme. As stated previously, appropriate concentrations of 5-HT are essential for signaling, and alterations in these concentrations, as produced by SSRIs like Zoloft could disrupt this signaling process and cause abnormalities in the lip and/or palate structures.

Further evidence that 5-HT is involved in craniofacial morphogenesis is provided by a study of mouse embryos in whole embryo culture. In this culture system, embryos were shown to grow normally at rates approximating those observed in utero (Sadler, '79), and they also responded to teratogens in a manner that simulates responses to these chemicals in whole animals (Sadler et al., '82; '84). When SSRIs including Zoloft were added to this model system at the time that facial prominences were developing and beginning their differentiation (3-6 weeks of human development), craniofacial malformations were produced in the tissues adjacent to 5-HT uptake sites, including the nasal and maxillary prominences (Shuey et al.,' 92). Furthermore, the effects were consistent with Wilson's Principles of Teratology in that they were dose and stage dependent, thereby supporting the validity of the results. The studies also revealed that SSRI exposure reduced proliferation and increased cell death in mesenchymal cells in craniofacial processes that ultimately became malformed, which indicates that normal epithelial-mesenchymal signaling, essential for normal development, had been disrupted by these treatments. (Shuey et al., '92).

D. SSRIs Including Zoloft Interfere with Palatal Shelf Re-orientation

In addition to being involved in NCC differentiation and epithelial-mesenchymal interactions essential to normal lip and palate development, 5-HT has been shown to play a role in later stages of palate morphogenesis when palatal shelves are re-orienting in preparation for fusion in the midline. 5-HT is present in palatal shelves prior to and during their re-orientation from their vertical position beside the tongue to their horizontal position in preparation for fusion (Wee et al., '79; Zimmerman et al., 81). Moreover, the addition of 5-HT to palatal shelves in culture stimulated anterior rotation of the shelves, which is important for their re-orientation. Importantly, serotonin antagonists inhibited such rotation (Zimmerman and Wee, '83). The

mechanism for rotation appears to lie in contractile elements in palate mesenchyme cells whose movement in cell culture is dependent upon stimulation of such elements by 5-HT (Venkatasubramanian and Zimmerman, '83). Similar effects of 5-HT on contractile elements and cell motility have been demonstrated in other cell systems. For example, 5-HT, via signaling through its receptors, has been shown to form and reorganize actin filaments that are known to play a prominent role in cell contractility and movement (Mineau-Hanschke et al., '89; Day et al., '06; Gill et al., '08). These studies elucidate the vital role 5-HT plays in palate shelf re-orientation. Therefore, if 5-HT concentrations are altered by SSRIs like Zoloft, palatal clefting defects could result based on the effects on cell contractility that promote the essential movement of the shelves that cause their re-orientation.

In summary, SSRIs like Zoloft can perturb each of the above-referenced key craniofacial developmental processes resulting in congenital cleft lip and palate defects.

VIII. Flat Bones and Sutures of the Skull and SSRI (Zoloft)-induced Malformations

A. Normal development of the flat bones and sutures of the skull

The flat bones of the skull encase the brain and differentiate directly from mesenchyme by membranous ossification. Neural crest cells (NCC) form the frontal and parts of the sphenoid and temporal bones, while paraxial mesoderm forms the parietal, occipital, and part of the temporal bone (Fig. 12). Sutures are fibrous joints that form between bones of the skull that allow molding of the head to facilitate passage through the birth canal and for continued growth of the skull in coordination with growth of the brain (Fig. 12; Adapted fro*m Langman's Medical Embryology*). Coronal sutures separate the frontal bones, while the sagittal suture separates the parietal bones (Fig. 12). NCC form the sagittal suture and mesoderm forms the coronal sutures (Morriss-Kay et al., '05). Thus, these sutures establish boundaries between different cell types, which serve as tissue organizers and signaling centers for growth of the skull. (Dahmann and Basler, '99; Opperman, '00; Jiang et al., '02). Through signaling pathways, these centers regulate cell proliferation and differentiation that, in turn, maintain normal growth and development of the sutures (Jiang et al., '02; Morriss-Kay, et al., '05). Thus, NCC and mesoderm cells are essential for proper formation and development of the skull (Merrill et al., 06; Komatsu et al., '13). Meninges (coverings of the brain) are also important in the process of bone formation and

maintenance of sutures, particularly the dura mater, which is NCC derived and regulates bone differentiation (Opperman, '00; Opperman et al., '95,'98; Jiang et al., '02).

Disruption of signaling pathways involving NCCs (Komatsu et al., '13) or in boundary cells around the sutures (Merrill et al.' 06) can result in premature loss of growth centers within the sutures causing ossificiation, and attendant craniosynostosis. Similarly, disruption of signals within the meninges (dura mater) can also result in early closure of the sutures and craniosynostosis (Opperman et al., '98; Opperman, '00). Thus, NCCs play an essential role in maintaining normal suture growth and in the origin of craniosynostosis.

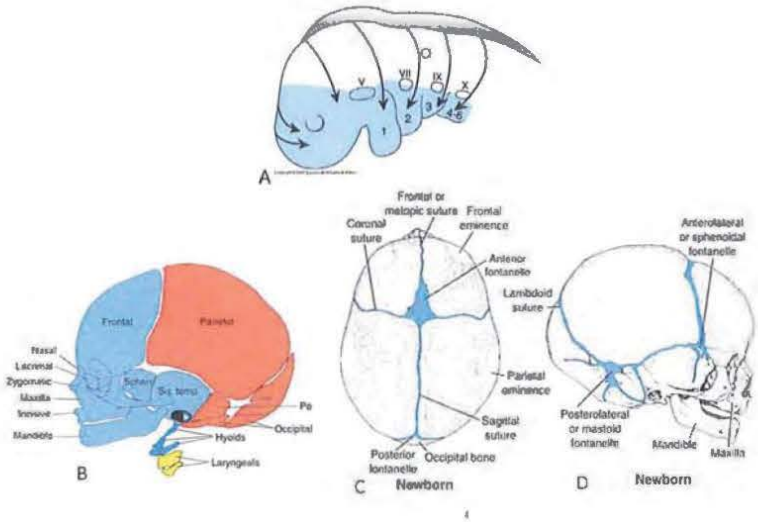


Figure 12. Formation of the cranial bones and sutures. **A.** Neural crest cells (NCC) migrate from the neural folds into the craniofacial region (Arrows) to form bones of the face and skull. **B.** Newborn skull showing that bones of the face and some in the skull are derived from NCC (Blue); whereas the remainder of the skull is derived from paraxial mesoderm (Red). **C.** Dorsal view of the sutures in a newborn's skull. The coronal and sagittal sutures are particularly important as signaling centers regulating growth of the skull. **D.** Lateral view of the sutures in a newborn's skull.

B. SSRIs Including Zoloft Can Cause Craniosynostosis

Craniosynostosis is a birth defect in which one or more of the fibrous sutures in an infant's skull prematurely fuses by forming bone (ossification) thereby changing the growth pattern of the skull. As was discussed previously in Section **VII** and above, during embryogenesis, NCC normally migrate from the neural folds into the craniofacial region to form the facial prominences, important for lip and palate development, as well as the bones of the face

and some bones in the skull. NCCs also form the meninges which cover the cerebral hemispheres. NCC have serotonin receptors (Moiseiwitsch and Lauder, '95; Choi, '97) and express the 5-HT transporter SERT (Hansson et al., '99). Serotonin regulates NCC migration and viability (Moiseiwitsch and Lauder, 95; Choi et al., '97). Because these cells participate in formation of the cranial sutures and dura mater, which serve as important signaling centers for suture differentiation (as described above), SSRI mediated changes in 5-HT concentrations could perturb normal NCC differentiation and/or signaling, and thereby cause craniosynostosis.

IX. Neural Tube Development and SSRI (Zoloft)-induced Malformations

A. Normal Neural Tube Development

Neural tube development begins on approximately day 19 of gestation with induction of the neural plate in the cranial region (Fig. 13A). Ectoderm cells in the region change from a flat cuboidal shape to a tall columnar profile characteristic of a placode. A day later, the neural plate begins to elevate at the edges (Fig.13B), forming the neural folds, and eventually these folds move toward the midline (Fig. 13C). The process begins in tbe cranial region and progresses caudally, such that neural folds forming the brain will develop first followed sequentially by folds in more caudal regions, including the spinal cord. Closure of the folds to form the neural tube commences in the cervical region and continues in zipper-like fashion both cranially and caudally (Fig. 13C and D). Additional closure sites appear in some parts of the cranial folds, such that closure is slightly different in this region. Cranial neural folds close by day 25 of gestation, while the caudal (spinal cord) end of the tube closes by day 28 (Fig.13E; Adapted from *Langman's Medical Embryology*).

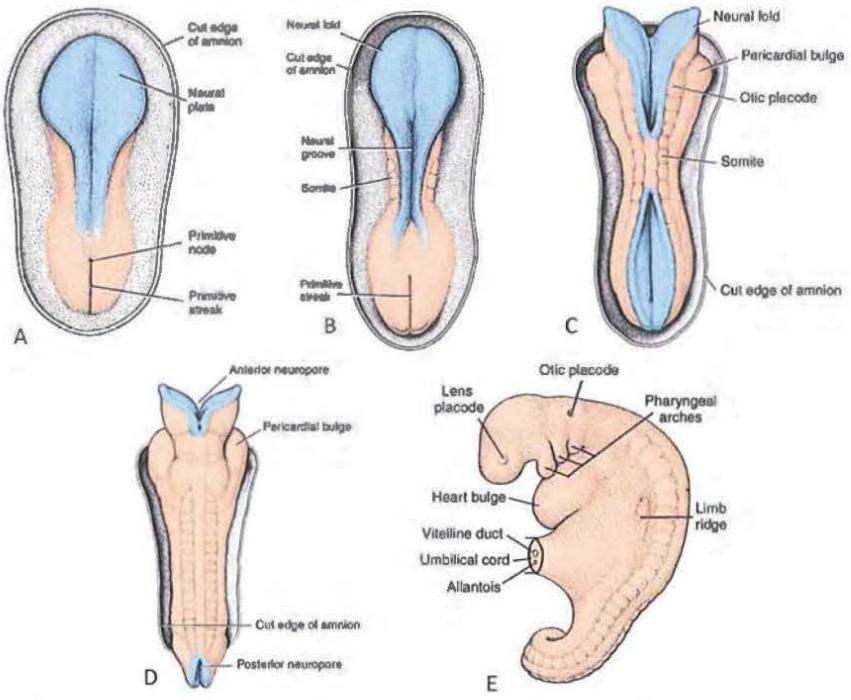


Figure 13. Neurulation: Formation and closure of the neural tube. **A.** Day 19: Thickening of the surface ectoderm forms the neural plate (blue) in the cranial region; **B.** Day 20: Edges of the plate begin to elevate and create the neural folds; **C.** Day 22: Neural folds initiate closure in the cervical region and then zipper cranially and caudally as they close the neural tube; **D.** Day 23: Most of the neural tube has been formed by closure of the neural folds except at the cranial and caudal openings, called the anterior and posterior neuropores, respectively; **E.** Day 28: By this time, closure of the neural tube is complete.

Neural tube defects (NTDs) arise when there is a lack of closure between opposing neural folds (Smith and Schoenwolf, '97; Sadler, '98; '05; Colas and Schoenwolf, '01). If closure fails in the cranial region it results in defects called encephalocele and anencephaly; failure to close in caudal regions results in various forms of spina bifida. A multitude of cellular factors play a role in the complicated process of neural fold formation and closure, including lengthening the tube by convergent extension (Schoenwolf and Alvarez, '89; Keller et al., '92; '00; '02); contraction of actin filaments and the employment of other cytoskeletal elements to move the folds (Nagale and Lee, '80; Sadler et al., '82; Lee and Nagale, '85); differing rates of cell proliferation in specific locations in the tube to promote bending (Smith and Schoenwolf, '88; '89); changes in extracelluar matrix components to facilitate initial stages of fold elevation (Solursh and Morriss-Kay, '77; Morriss-Kay and Crutch, '82); and others (For reviews see Smith and Schoenwolf, '97; Sadler, '98; '05; Colas and Schoenwolf, '01). Disruption of any of these processes can result in a NTD.

B. SSRIs Including Zoloft Can Cause Neural Tube Defects

The 5-HT2 class of receptors are present in the neural folds during neural tube closure (Choi et al.' 94; Lauder et al., '00), and exposure of mouse embryos in whole embryo culture to receptor antagonists resulted in NTDs (Lauder et al., '00). In a similar culture system, mouse embryos grown in the absence of 5-HT, all developed NTDs (Roux et al., '95), while chick embryos exposed to chemicals that alter 5-HT concentrations in the embryo also resulted in NTDs (Palen et al., '79).

Taken together these studies provide important evidence demonstrating serotonin's essential role in neural tube closure. One mechanism by which 5-HT is likely involved in neural tube closure is via regulation of cell proliferation, essential for closure of the neural folds. Studies have shown that the 5-HT2B receptor regulates proliferation of cells in culture (Lee, et al., '91; 99; Buznikov et al., '01), while the 5-HT2A and 5-HT2C receptors regulate mitogenic effects on fibroblasts in culture (Julius et al., '89). In each of these studies, 5-HT signaling through its receptors is dependent upon appropriate 5-HT concentrations. If the concentration of 5-HT is disrupted, as occurs with the use of SSRIs like Zoloft, signaling will be abnormal.

Another mechanism whereby 5-HT is likely involved in neural tube closure is through its effects on the cytoskeleton. As mentioned above, actin and other elements of the cytoskeleton are essential to provide bending of the neural folds toward the midline (Nagale and Lee, '80; Sadler et al., '82; Lee and Nagale, '85; Sadler et al., '86). Signaling by 5-HT through its receptors has been shown to form and reorganize actin filaments in a dose dependent manner (Mineau-Hauschke, '89; Day et al., '06; Gill et al., '08). If such a mechanism is operating in neuroepithelial cells during neural tube closure, alteration of 5-HT concentrations, as produced by SSRIs like Zoloft, would disrupt 5-HT signaling and the molecule's capacity to regulate the cytoskeleton, which could induce neural tube defects.

In addition to 5-HT receptors in the neural folds, there are also uptake sites for 5-HT (SERT) in the cranial and caudal ends of the neural tube (Wallace, '82). In the brain, these sites are localized to the floor plate in regions where flexures occur and the neurotransmitter may be involved in signaling for that process (Wallace, '82). In the caudal region of the neural tube, uptake sites are localized to the floor plate and notochord and these sites are transient, such that, once closure occurs in one segment of the caudal neural tube, the sites disappear in that location and move further caudally where closure is still occurring (Wallace, '82). Coordination of these

uptake sites with closing regions of the neural tube indicate that 5-HT plays a role in the closing process via mechanisms described previously (cell proliferation, cytoskeleton).

Other roles for 5-HT signaling in the caudal region of the neural tube that would affect neural tube closure and cause neural tube defects are related to the phenomenon called convergent extension that is essential for normal neural tube development (Schoenwolf and Alvarez, '89; Keller et al., '92; '00; '02; Ybot-Gonzalez et al., '07). Convergent extension is the process whereby the medio-lateral intercalation of cells lengthens and narrows the neural plate (Keller, '02). The process is regulated by the planar cell polarity pathway (PCP; Wang et al., '06; Simons and Mlodzik, '08; Henderson and Chadhry, '11) and 5-HT appears to be involved in the signaling process through its effects on the 5-HT2 receptor that regulates cell junctions and the cytoskeleton (Colas et al., '99b). Studies show that a peak of 5-HT concentration that coincides with the expression of the 5-HT2 receptor is required for normal convergent extension to occur (Colas et al., '99a) and gain of affinity mutations in the receptor accelerate the convergent extension process (Schaerlinger, et al. '07). Also, it is known that serotonergic neurons use the planar cell polarity pathway (PCP) that regulates convergent extension as these neurons migrate to their proper location in the brainstem (Fenstermaker et al., '2010). The phenomena of convergent extension and PCP signaling are so important to normal embryonic development, that the processes have been conserved from fruit flies to mammals (Wang et al., '06; Simons and Mlodzik, 08). Thus, 5-HT may regulate the important process of convergent extension essential to normal neural tube closure (and for lengthening the outflow tract of the heart[Section **VI,** parts 1 and 2] and for gastrulation Section **X,** part 2). Indeed, in a similar scenario, neural tube defects have been observed in mice and humans when genetic signaling regulating convergent extension has been disrupted (Murdoch et al., '01;Wallingford and Harland, '02; Doudney and Stanier, '05; Wang et al., '06; Kibaret al., '07; '11; Torban et al., '08; Lei and Zang, '10).Thus, 5-HT may regulate the important process of convergent extension essential to normal neural tube closure.

In summary, the fact that 5-HT concentrations are critical for proper signaling necessary for convergent extension (Colas et al., '99; Schaerlinger, et al. '07) indicates that SSRIs like Zoloft that alter 5-HT concentrations, could cause neural tube defects by inhibiting this essential cell process. It has also been proposed that altering 5-HT signaling in this process as would occur by exposure to SSRIs like Zoloft could render the neural tube more susceptible to a teratogenic insult that would cause a neural tube defect. (Colas and Schoenwolf, '01).

X. Gastrulation: A Target for SSRIs Like Zoloft: Vertebral and Urogenital Defects

A. Normal Embryology of Gastrulation

As discussed in Section **IX,** convergent extension is an important cell process for normal neural tube development. It is also essential for gastrulation, a movement of cells that establishes the germ layers in the embryo and that also lengthens the embryonic axis (Keller, '02). During gastrulation, cells in the dorsal layer (epiblast) of the embryo move toward a depression in the epiblast in the caudal region called the primitive streak (Fig. 14A), detach from their neighbors, and migrate beneath the remaining epiblast cell layer (Fig. 14B), while also displacing cells in the hypoblast (the ventral layer of the bilaminar embryo; Fig. 14B). In so doing, 2 new layers are created: a middle layer, called the mesoderm; and a ventral layer called the endoderm. Some cells remain in the epiblast to form the ectoderm.

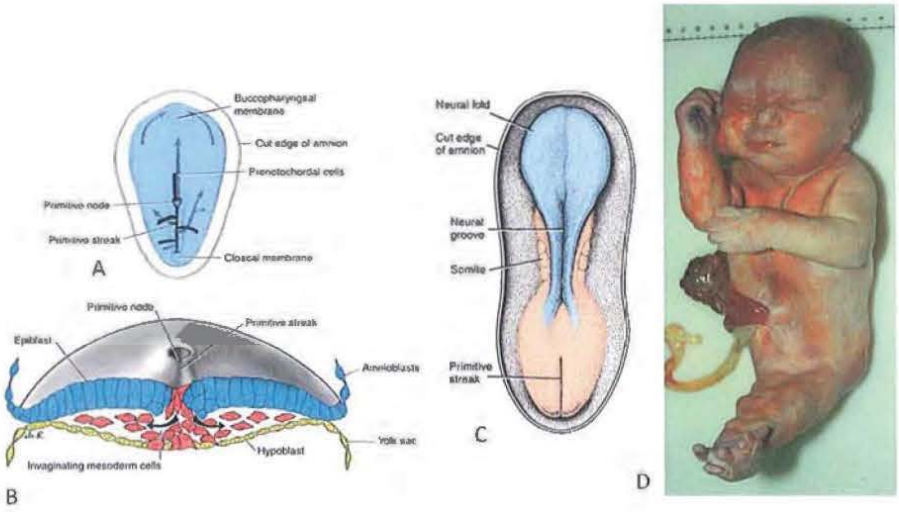


Figure 14. Gastrulation. **A.** Day 16: Dorsal view of an embryo at the bilaminar disc stage (oreo cookie without icing) showing movement of cells in the top layer (epiblast; blue) moving toward the primitive streak, a depression at the caudal end of the embryo. Cells turn in at the streak, detach, and migrate beneath the epiblast layer. **B.** Cross section through the primitive streak of a day 16 embryo showing epiblast (blue cells, top layer) moving into the streak and detaching to form a middle layer of cells (icing layer for the cookie) called the mesoderm. Some of these detached cells also displace the hypoblast (yellow cells) to form a new ventral layer called the endoderm. Other cells remain in the epiblast and these form the ectoderm. Ectoderm, mesoderm, and endoderm comprise the 3 germ layers that will "germinate" all the tissues and organs for normal development. **C.** A day 20 embryo showing that, while neurulation (see Section IX, Figure 12) is occurring in the cranial region, gastrulation is still taking place through the primitive streak at the caudal end. In fact, at this stage in this embryo, gastrulation has created sufficient numbers of

germ layer cells to form only the head region. **D.** Fetus with caudal dysgenesis. Gastrulation was inhibited in this baby at a stage slightly later than the embryo shown in **C** and as a result there were not enough cells to form the lower part of the body. There are no kidneys, severe urorectal defects are present, and the lower limbs are fused, giving the condition its name, sirenomelia or Mermaid's syndrome.

Gastrulation starts in the cranial region and proceeds caudally, transforming the bilaminar embryo (in the shape of an oreo cookie without icing) into a trilaminar embryo (oreo cookie with icing; Fig. 14). In so doing, the process provides for all of the cells to form organs and tissues from the brain and skull to the anorectal region. The 3 layers are called the germ layers because they "germinate" all the tissues and organs in the embryo. Ectoderm forms skin and sensory organs, like the brain and spinal cord. Mesoderm forms the heart, urogenital system, and bones. Endoderm forms the gut and all its derivatives.

If gastrulation is interrupted, caudal regions fail to develop and the resulting loss of structures is dependent upon the stage at which the interruption occurred. A severe example of such a failure is called sirenomelia, also known as Mermaid's syndrome (Fig. 14D). With sirenomelia, gastrulation is inhibited in early development, such that while the head, upper limbs, and thorax develop as expected, the abdominal region, including the kidneys, and other more caudal structures do not have sufficient cells to form normally. In addition to absent kidneys, the lower limbs are fused, there are urogenital defects, and caudal segments of the vertebral column are absent. Such a collection of malformations are part of a constellation of abnormalities called caudal dysgenesis. The severity of caudal dysgenesis varies; sirenomelia is at the severe end of the spectrum, whereas missing sacral vertebrae, imperforate anus and urorectal defects are at the other (Adapted from *Langman's Medical Embryology,* 12th ed.).

C. Gastrulation and SSRI (Zoloft)-induced Malformations

A key component of gastrulation is convergent extension (See section **IX**). Inhibition of this process via manipulation of genes responsible for regulation of cell movement results in shortening of the craniocaudal body axis, vis-à-vis, caudal dysgenesis (Park and Moon, '02; Topczewski et al., '02; Veeman et al., '03; for review, see Ueno and Greene, '03). As mentioned previously, 5-HT is present in the caudal region of the embryo at the time of gastrulation (Buznikov, et al., '64; 72; Wallace, '82; Colas, '99a) as are its receptors (Colas, 99b) and both have been shown to play a role in regulating convergent extension during gastrulation (Colas,

'99a,b). In fact, studies show that gastrulation can be disrupted by misexpressing 5-HT receptors or by changing 5-HT concentrations (Colas, '99a,b). Since SSRIs like Zoloft are designed to alter 5-HT concentrations, they have the potential to interfere with gastrulation and cause caudal regression type defects. The stage of development and concentration of SSRIs would dictate the type and severity of defects that would occur ranging from missing lumbar and sacral vertebrae with urorectal malformations (anal atresia, rectovaginal fistulas) at one end of the spectrum to more severe caudal defects at the other. Interestingly, these types of defects are commonly observed in cases of heterotaxy and may occur as isolated defects in families with abnormal L-R patterning (see Section V; Kasaki and Casey, '98; Casey, '98).

XI. Limb Development as a Target for SSRIs Like Zoloft

As mentioned in Section **V**, normal limb development is related to establishing normal laterality in the embryo and many limb defects arise from alterations in that process, including aplasias, dysplasias, and clubfoot. Since 5-HT plays a key role as a signaling molecule in the pathway that specifies laterality, alterations in 5-HT concentrations, as produced by SSRIs like Zoloft could cause limb defects.

In addition to its role in specifying patterning for the limbs, 5-HT also plays a role in limb chondrogenesis. Normally, limb bud mesenchyme is maintained in a proliferative state by the apical ectodermal ridge (AER; Fig. 15). As the limb grows outward, proximal mesenchyme cells become located at greater distances from the AER and their proliferative rates decrease in preparation for differentiation into cartilage (Fig. 15). Signaling for these events is mediated by retinoic acid, secreted from the flank of the embryo, and fibroblast growth factors (FGFs), secreted by the AER, such that there are overlapping diffusion gradients along the proximodistal limb axis. Cells close to the flank are exposed to high concentrations of retinoic acid and low concentrations of FGFs and so they begin to differentiate into cartilage, while cells at the distal end are exposed to high concentrations of FGFs and low concentrations of retinoic acid, causing these cells to remain undifferentiated and rapidly proliferating (Benazet and Zeller, '09). As cells differentiate, they condense into specific cartilaginous regions, each of which forms a model for the bone in that region. In this manner each bony limb component is modeled in cartilage first and then ossification occurs (Fig.15). Such a process is called endochondral bone formation. If insufficient numbers of mesenchyme cells are formed or if the cartilage models themselves do

not form properly due to abnormal differentiation of chondrogenic precursors, then defects in the bones occur. These defects may include aplasia and dysplasia type defects, as well as clubfoot. (Adapted from: *Langman's Medical Embryology,* 12th ed.).

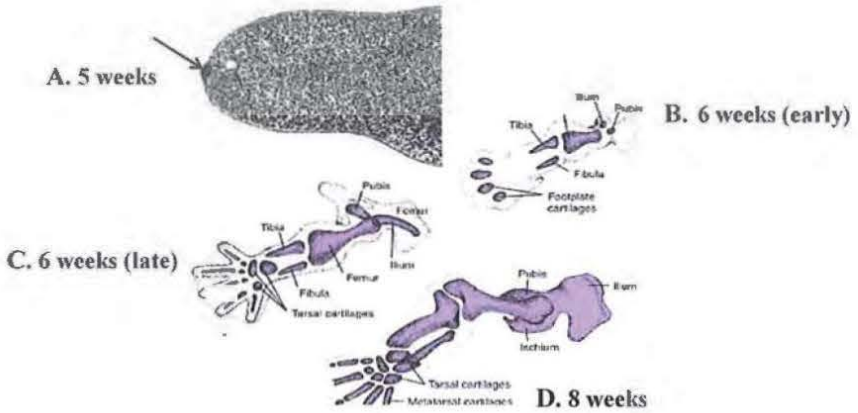


Figure 14. Limb development: Formation of cartilage. **A.** Histological section of a limb at 5 weeks showing the apical ectodermal ridge (AER; arrow) at the tip of the limb and mesenchyme cells throughout the limb bud. **B.** Between 5 and 6 weeks, cartilages begin to differentiate in proximal segments and soon extend all the way to the foot plate. **C.** At 6 weeks, cartilaginous precursors are present for most of the bones of the limb. **D.** By 8 weeks, all of the cartilage rudiments are present, including those of the foot (and hand).

Studies have shown that 5-HT can regulate the process of cartilage differentiation. For example in craniofacial mesenchyme cell cultures, 5-HT receptor antagonists have been shown to down-regulate cartilage matrix production (Moiseiwitsch and Lauder, '97) and to decrease cell proliferation in these cultures by signaling through 5-HT receptors that activate the cyclic adenosine monophosphate/protein kinase A (CAMP/PKA) pathway (Lambert and Lauder, '99; Lambert et al., '01). Acting through other 5-HT receptors, the neurotransmitter stimulates proliferation in these mesenchyme cells (Buznikov et al., '01). Similar types of regulation by 5-HT and its receptors are observed in hindlimb bud mesenchyme cell cultures (Bhasin et al.,'04). If concentrations of 5HT are disrupted, as occurs with SSRIs like Zoloft, 5-HT signaling, important for proliferation and differentiation of chondrogenic cells in the limb, would be adversely affected and lead to limb abnormalities, including aplasias, dysplasias, and clubfoot.

One limb defect whose etiology has been linked to abnormal cartilage formation is clubfoot. In a study, the clubfoot calcaneum was found to have fewer chondrocytes and a smaller

number of cartilage canals. Also, zones of differentiated chondrocytes were absent from the growth plate region (Gilbert et al., '01). These cellular defects are characteristic of the types resulting when 5-HT signaling was disrupted in these tissues as occur if 5-HT concentrations were altered as they are by SSRIs like Zoloft.

XII. The Link between SSRIs Like Zoloft and Omphalocele

During normal development, the gut tube herniates into the umbilical cord at approximately the 6th week of development. The primary loop leads the way and rotates 90° counterclockwise. As the bowel lengthens, more of it enters the cord. During the 10th week, the gut begins to return to the abdominal cavity and as it returns it rotates an additional 180°. This places the various parts of the gut into their proper position on the posterior body wall. The process is called physiological herniation of the gut (adapted from: *Langman's Medical Embryology,* 12th ed.).

Mechanisms that regulate the process are poorly understood, however, because the rotation that is essential to place the gut in its proper location, coupled with the fact that regions of the gut must be specified for the right and left sides of the abdomen, i.e. ascending colon to the right, descending colon to the left, it appears that laterality signaling is involved. As mentioned in Section **V**, 5-HT is a key signaling molecule involved in establishing left-right asymmetry in the body and therefore, could be involved in the origin of omphalocele. The fact that omphalocele is observed in families and individuals with laterality defects supports this conclusion (Martinez-Frias et al., '95; Ticho et al., '00; Ware et al., '04; Boe et al.,'08). It is also possible that 5-HT is more directly involved in the process of physiological umbilical herniation by regulating gut motility or some other mechanism. Support for this hypothesis is derived from animal studies showing that 5-HT is a teratogen and one of the defects that it causes is omphalocele (Reddy et al., '63; Van Cauteren et al. '86: See Section **XIII**). This result indicates that too much 5-HT interferes with the herniation and return process. The fact that SSRIs like Zoloft alter 5-HT concentrations suggests that these changes in concentration are likely involved in the origin of this defect.

XIII. The Link Between SSRIs Like Zoloft and Gastroschisis

Gastroschisis is a defect that arises attendant to abnormalities in closure of the ventral body wall (Feldkamp et al. '07; Sadler and Feldkamp, '08). Normally, lateral body wall folds form during the 3rd and 4th weeks of development and grow ventrally toward the midline where they approach each other and fuse (Fig. 16) in a process somewhat similar to neural tube formation (Sadler and Feldkamp, '08; See Section **IX**). The fusion process is complex as different cell layers must establish a connection with each other across the midline and some layers must detach from others (Figs. 16 and 17; Feldkamp et al., '07; Sadler and Feldkamp, '08). Gastroschisis occurs when fusion fails or if lateral body wall folds fail to grow sufficiently to meet each other at the midline. In either case, a hole or gap remains in the body wall and abdominal contents (usually loops of bowel) protrude through the opening.

In 95% of cases, the defect occurs on the right side, thereby supportive evidence that laterality signaling plays a role in specifying the lateral body wall folds (Fig. 17C; Feldkamp et al., '07). This proposal is also supported by the observation that mice in which there is a deficiency of the key laterality gene *Pitx2* have gastroschisis (Kitamura et al., '99). Because 5-HT is important for laterality signaling (Fukumoto et al., '05a,b), alterations in 5-HT concentrations, as are produced by SSRIs like Zoloft could disrupt development of the body wall folds resulting in gastroschisis.

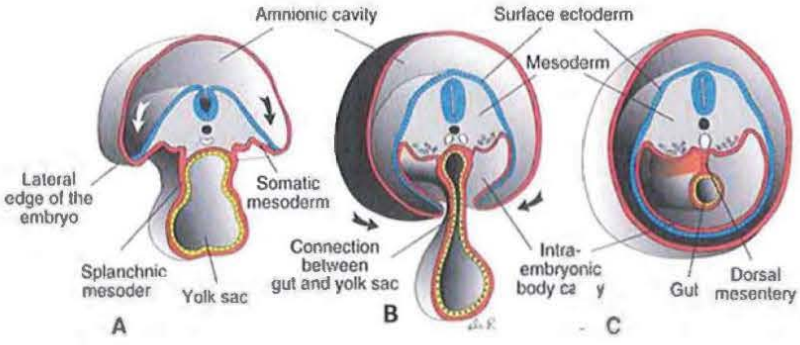


Figure 15. Cross sections through embryos at the time of body wall formation and closure. **A.** At approximately 21 days of gestation, lateral body wall folds (arrows) are well formed and are moving ventrally. **B.** By 25 days, the folds are approaching each other in the midline and the opening between the gut tube and yolk sac is narrowing. **C.** By 28 days, closure is complete and the two folds have fused in the midline. Note the reorganization of cell layers after closure and that the embryo is now surrounded by the amniotic cavity.

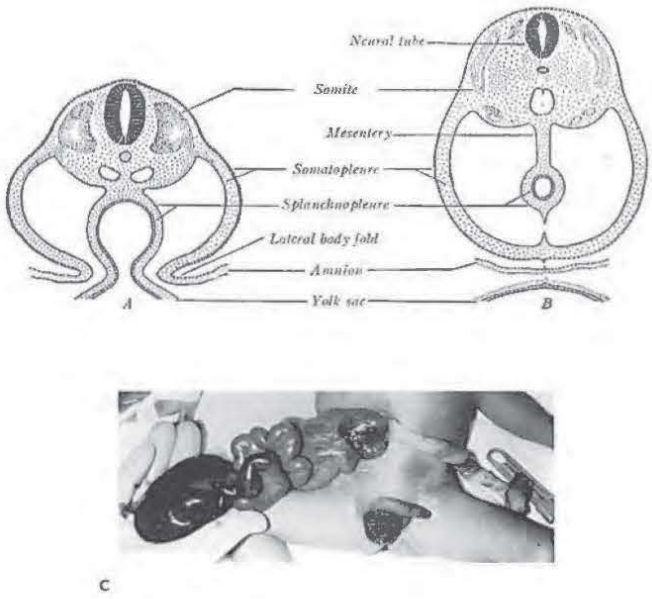


Figure 16. Cross sections through embryos during closure of the lateral body wall folds. **A.** The folds (somatopleure) approach each other in the midline. **B.** Fusion has occurred. Note how the body wall and amnion have reorganized to form complete cell layers across the midline. Also note that these cell layers have to separate from each other and that the gut tube (splanchnopleure) has to "pinch off" from the yolk sac. **C.** Newborn with gastroschisis. Loops of bowel are protruding from an opening to the right of the umbilical cord.

Another mechanism whereby 5-HT may be important for body wall folding is through the process of convergent extension, which is regulated by signaling through the planar cell polarity (PCP) pathway (Wang et al., '06; Simons and Mlodzik). As discussed above, convergent extension is the process that cells employ to move in a polarized direction to lengthen different structures like the body axis during neurulation (see Section **IX**) and gastrulation (see Section **X**), and the heart tube (see Section**VI**) and other structures. The phenomenon is regulated by the PCP and 5-HT has been shown to participate in this signaling pathway (Colas et al., 99a,b; Schaerlinger, et al. '07). The lateral body wall folds must also lengthen as they grow ventrally, and there is evidence that PCP signaling and convergent extension is involved in the process. For example, mice that develop neural tube defects due to mutations that regulate PCP signaling and convergent extension also have gastroschisis (Kibar et al., '01; Murdoch et al., '03; Wang et al., '06). Also, at least one of these genes has been shown to be expressed in the lateral body wall folds at the time they are moving ventrally toward the midline (Murdoch et al., '03). Thus, closure of the body wall may rely on convergent extension types of cell movements regulated by the PCP pathway that utilizes 5-HT as a signaling molecule. Changing 5-HT concentrations, as

occurs with SSRIs like Zoloft, could disrupt this signaling resulting in abnormal growth and development of the body wall folds and gastroschisis.

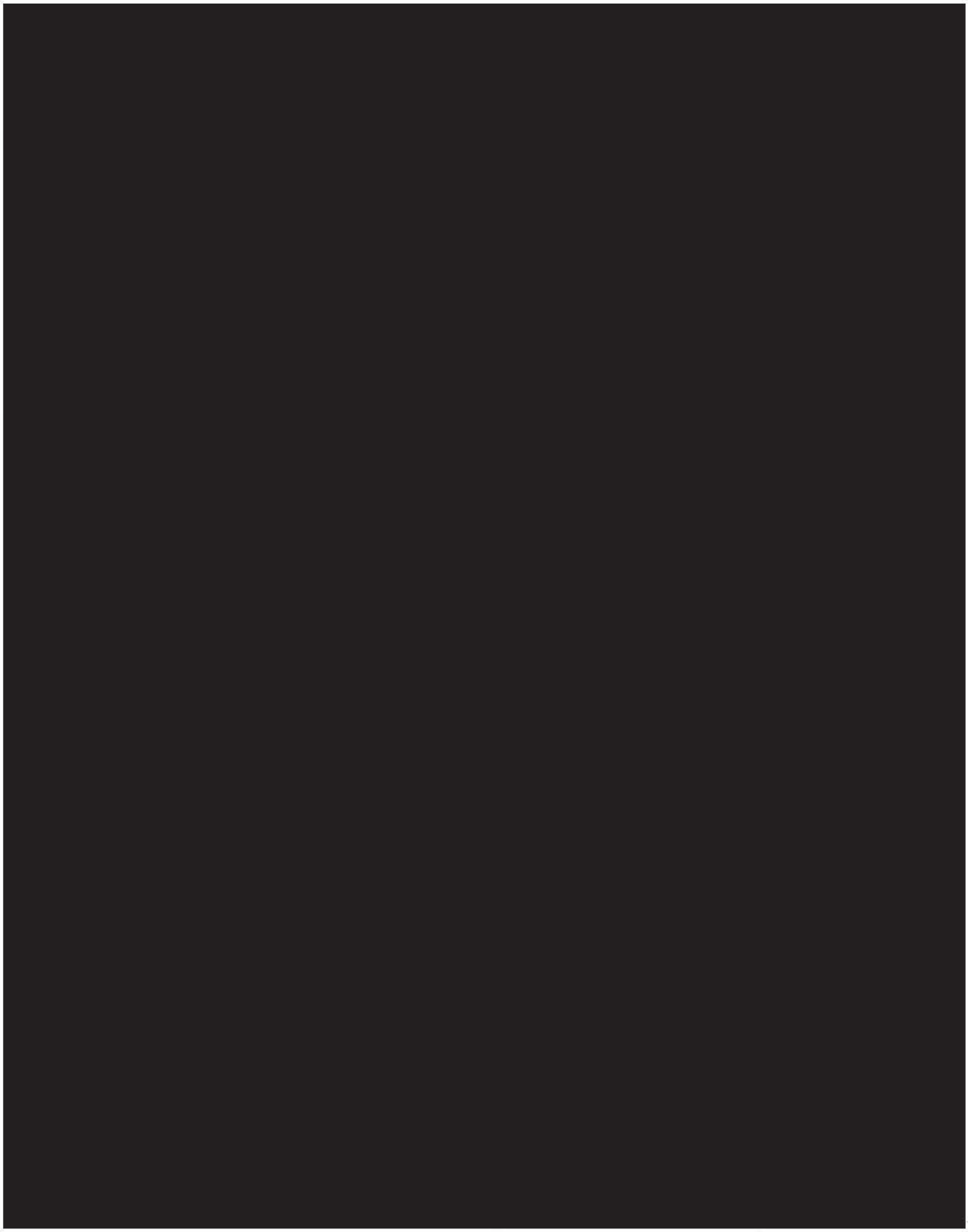
XIV. Serotonin and Zoloft as Teratogens and Abortifacients

A. Serotonin as a Teratogen and Abortifacient

Several animal studies have shown that 5-HT is teratogenic and causes abortions (resorptions) in a dose dependent fashion when administered during the period of embryogenesis (Poulson et al., '63; Reddy et al., '63; Marley et al., '67; Van Cauteren et al., '86). Birth defects observed include, neural tube defects, limb abnormalities, omphalocele, eye abnormalities, gastroschisis, cleft lip, and rib anomalies; in other words, malformations similar to many of those described in the previous sections. And, like those abnormalities described previously, 5-HT-induced defects in these teratology studies were due to abnormal concentrations of the neurotransmitter. In addition, there was an increase in pregnancy loss (resorptions) in these studies, an effect that has also been reported following the use of SSRIs (Baur et al., '10; Broy and Berard, '10; Domar et al., '12). Therefore, since SSRIs like Zoloft are designed to increase extracellular concentrations of 5-HT, there is a plausible link between the action of these drugs and the teratogenicity and abortifacient activity of 5-HT.

B. Zoloft as a Teratogen and Abortifacient

The preclinical animal studies conducted by Pfizer provide evidence of Zoloft's potential adverse effect on reproduction and embryogenesis. [redacted]



XV. Postscript

At first glance, it may appear as though 5-HT plays a role in every developmental event that takes place during embryonic development. However, closer examination of 5-HT signaling during development shows that the molecule is involved in a smaller number of phenomena, but that these phenomena are related to multiple developmental events. For example, 5-HT is important for establishing laterality and disturbing this process leads to multiple downstream abnormalities; it regulates neural crest cell behavior and these cells are linked to formation of many embryonic structures; it plays a role in convergent extension that is involved in gastrulation, neurulation, and extension of the outflow tract of the heart. These 3 functions of 5-HT alone are linked to many of the birth defects caused by SSRIs like Zoloft. Furthermore, it is not unusual for a signaling molecule or a family of signaling molecules to be involved in multiple developmental processes. Family members of the fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and transforming growth factor beta group (TGFβ) all participate in regulating formation and differentiation of many cell types and organs. Perhaps the best example of such a role played by a single molecule is that of sonic hedgehog (SHH). Signaling by SHH is involved in vasculogenesis, laterality, heart development, limb formation,

kidney morphogenesis, tooth differentiation, eye development, cranial neural crest patterning, and many other processes (Varjosalo and Taipale, '08). Thus, there is a precedent for 5-HT to function in many different cells and processes during embryogenesis, or, perhaps 5-HT set the precedent since it is an "ancient" signaling molecule (Lauder, '93; Azmitia, '01; Buznikov et al., '01).

Signaling molecules like SHH and 5-HT that regulate developmental events are called morphogens and their use in such a wide variety of functions is possible because cellular responses to the signal depend on the responding cells, the concentration of the morphogen, and the duration of exposure to the morphogen. Cellular response to the morphogen is fine-tuned by feedback signals that regulate the amount of signal sensed by responding cells (Varjosalo and Taipale, '08). That is why the concentration of a morphogen, like 5-HT, is so critical to normal signaling and why changes in that concentration can disrupt the signaling process. The concentration of 5-HT is normally regulated by serotonin binding protein, the serotonin transporter SERT, and other factors. The presence of SSRIs like Zoloft alters 5-HT concentrations and that is the mechanism whereby this class of drugs disrupts 5-HT signaling pathways essential for normal development.

XVI. Conclusion

It is my opinion, rendered to a reasonable degree of scientific certainty that gestational exposure to SSRIs including Zoloft causes or substantially contributes to congenital birth defects. Accordingly, it is my opinion that Zoloft, at all conventional doses, significantly increases the likelihood of congenital malformations.

XVII. Disclosures

The methodology and materials upon which I rely relied in formulating my opinions are generally accepted in the scientific community. My opinions contained herein are neither new nor novel. My opinions herein are expressed to a reasonable degree of scientific certainty. I will testify at trial regarding the matters and opinions proffered in this report, as well as items reasonably related to the opinions contained in this report herein.

I reserve the right to alter or supplement my opinions. Additionally, I reserve the opportunity to testify in my areas of expertise in response to the testimony of Defendant's opinion witnesses. I am being compensated in connection with this matter at my customary rate of $500 per hour.

The following is a list of the cases in which, during the previous ten years, I testified as an expert witness at trial or by deposition.

Case:
Law firm: Phillips and Paolicelli, LLP
State: New York
Deposition: 2/28/13
Trial: N/A
Case resolution: Pending

T.W. Sadler, PhD Date: 7/16/2013

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